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Part I: Movement Disorders Due to Drug and Disease

It is apparent that certain drugs exert an incapacitating effect on man through impairment of motor function. This laboratory has long been interested in disorders of motor function produced by disease and drug, as well as in investigating the normal control of movement.

In this section are described methods of study which we have developed or which are currently being developed. These techniques are designed to investigate two aspects of the problems: (1) the site of action and mechanism of the disorder, and (2) the quantitative measurement of the disability.

With specific reference to the assessment of drug action the qualitative studies are useful in pointing out differences in site and mode of action of related chemical compounds. Such data are useful in maximizing certain actions while minimizing others. The quantitative data are of importance in comparisons between similar drugs in terms of potency and duration of action. While such studies are helpful in quantifying obvious deficits in performance, they are crucial in demonstrating deficits of motor function which are so minimal as to elude detection by clinical assessment.

The techniques to be described measure motor function from four different viewpoints: 1. The motor system is assessed by considering it to be a series of steps beginning with volition and ending with muscular contraction. 2. The system is regarded as a "black box" without regard to its detailed organization. It is considered to be a servomechanism with an input and output. 3. Detailed attention is directed toward the servocontrol at the spinal cord level and peripherally.

SERIAL TREATMENT OF MOTOR PERFORMANCE

Figure 1 illustrates the serial steps in voluntary movement which are amenable to quantitative measurement. Volition (1) is transmitted via the pyramidal and extrapyramidal tracts to the lower motor neuron (2) The impulses from the lower motor neuron pool are transmitted by neuromuscular transmission (3) to the muscle which contracts (4).

Previous studies (Johns and Liljestrand, unpublished observations) established that the maximum force of contraction produced voluntarily equals the maximum force produced by tetanic nerve stimulation. This was established by comparing the isometric tension measured with a strain gauge (fig. 1 SG) in response to maximum voluntary effort with the tension developed in response to repetitive electrical stimuli applied to the nerve (fig. 1 S). This fact provided a convenient tool for assessing the motivation of the subject. Subjects who do not exert their maximal effort will develop "maximal" voluntary tension which is less than the maximum tetanic tension. While lack of motivation is the commonest

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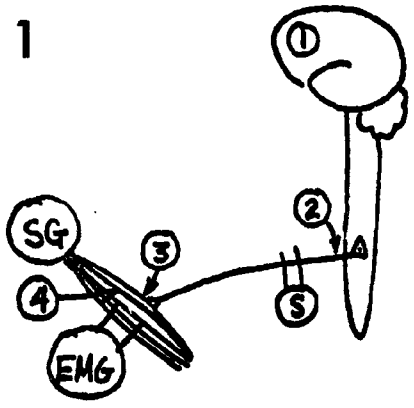
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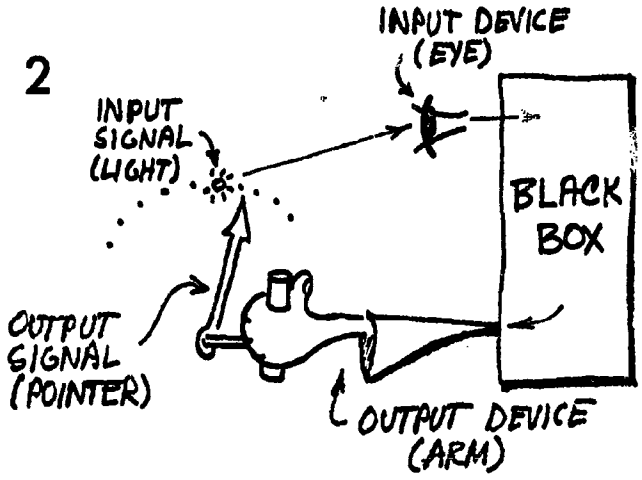
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INPUT SIGNAL (LIGHT)
BLACK BOX
OUTPUT SIGNAL (POINTER)
OUTPUT DEVICE (ARM)

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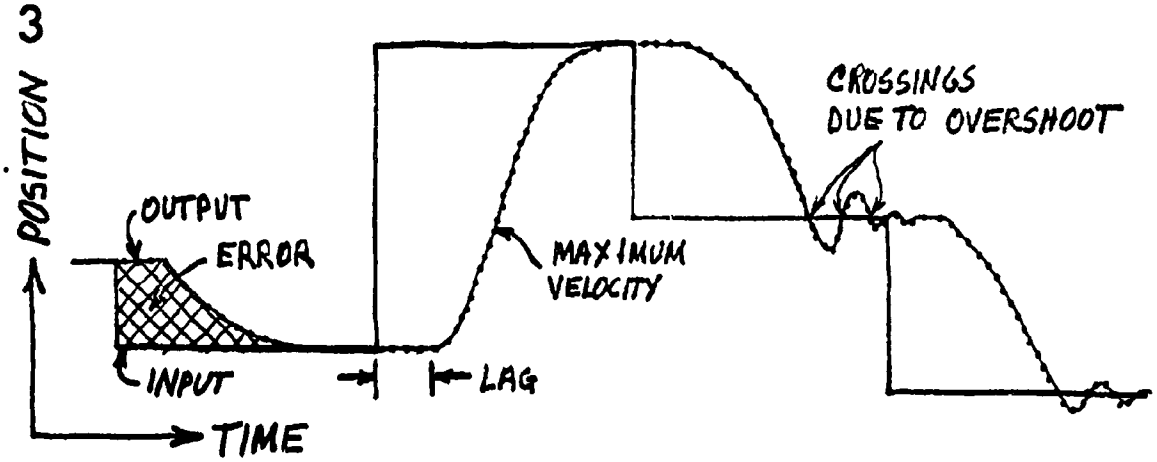
POSITION
TIME
OUTPUT
ERROR
INPUT
LAG
MAXIMUM VELOCITY
CROSSINGS DUE TO OVERSHOOT

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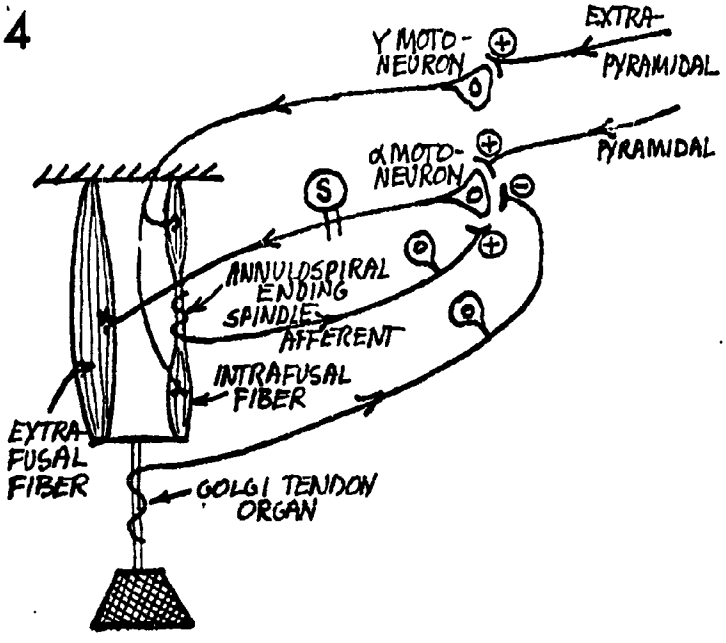
γ MOTO-NEURON
EXTRA-PYRAMIDAL
α MOTO-NEURON
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ANNULOSPIRAL ENDING
SPINDLE-AFFERENT
INTRAFUSAL FIBER
EXTRA-FUSAL FIBER
GOLGI TENDON ORGAN

cause of such a finding, any impairment of function above the lower motor neuron would be reflected as a reduction in maximum voluntary contraction in relation to the maximum tetanic tension in response to nerve stimulation.

Dysfunction of nerve can be discerned by measurements of nerve conduction velocity. This is done by measuring the difference in latency between the action potentials elicited. Fortunately, disorders in which there is failure of nerve conduction are associated with a slowing of the conduction velocity in the fibers less severely affected.

Neuromuscular transmission is measured quantitatively by determining the size of the muscle action potential in response to a supramaximal nerve stimulus. Reductions in amplitude of the muscle response are due to failure of neuromuscular transmission at some junctions. The electrical response measured with surface electrodes provides a convenient method of estimating this effect.

Reduction in the isometric tension in response to nerve stimuli in the absence of changes in neuromuscular transmission indicates some impairment of the contractile process of muscle. By this technique disorders of muscle contractility can be differentiated from problems in neuromuscular transmission. Furthermore, conventional needle electromyography serves as a useful tool for distinguishing between neuropathic and myopathic reductions in muscular strength. In the former, there is a reduction in the number of motor units responding, but those motor units which do respond are quite normal. In the latter, the number of motor units is not reduced, but the configuration of the motor unit potentials is abnormal due to abnormalities of the individual muscle fibers within the motor unit.

In summary, techniques presently in use in this laboratory are capable of localizing disorders of motor performance along the neuromuscular axis. These techniques have been applied to the study of abnormalities of motor function produced by disease. At present attention is turning to similar studies in which motor function is altered by drugs.

CONSIDERATION OF MOTOR FUNCTION AS A GENERALIZED SERVOMECHANISM

In this approach to the problem little emphasis is placed on the detailed means by which the human organism controls motor function. The system is studied from the standpoint of its input and output. The detailed mechanism is considered to be enclosed in a "black box" (fig. 2). The input is the means by which information concerning the specific task to be performed is transferred to the subject, while the output device is the mechanical means by which the task is performed. Servomechanisms such as this may be studied by variety of methods. Their performance may be characterized by measuring gain as a function of frequency of input signal, by measuring gain as a function of a phase shift

between input and output, or by characterizing its response to a step (abrupt change) in input.

It was this last technique which we chose, largely on the basis of simplicity of apparatus. The task which the subject was to perform was a visual tracking problem. A series of twenty lights were arranged in an arc of 95°. A single light would be illuminated in a serial and random fashion. The interval at which switching from one to another would occur was varied between one and four seconds. Mounted at the center of the arc was a "T" shaped handle to which a pointer was attached (fig. 2). The subject was instructed to keep the pointer directed toward whichever light was illuminated. Thus, the input to the subject was the random step in light position. Similarly, the output was the rotation of the pointer produced by the subject.

Electrical potentials were developed which were proportional to the input position and to the output position. Figure 3 illustrates schematically these signals. The input signal can be seen to be a series of steps of random amplitude. If the subject were a perfect tracking device the output signal would be super-imposed upon the input signal. Since the subject is not a perfect tracking device, the output signal does not correspond precisely to the input signal. The difference between these two (represented by the cross hatched area) is the error. Preliminary studies in normal subjects and patients with parkinsonism revealed that the error has several components. One contributor to error was lag. As can be seen in figure 3 there is a delay between the change in input position and initiation of the change in output position. This lag is the reaction time of the subject. In our present device these lags are recorded cumulatively.

A second contributor to error was the maximum velocity which the subject can achieve in tracking. This is represented by the maximum slope of the output signal. It is apparent that limitations in maximum velocity of movement will contribute significantly to total error. By a simple differentiation of the output signal maximum velocity was recorded separately.

A third type of contribution to error was seen in exaggerated form in the ataxic patient who exhibited damped oscillations in attempting to track the mark. This overshooting and correcting can be quantified by recording the number of times that the output signal crosses over the input signal. This, too, was recorded separately.

With this relatively simple device we were able to measure the total cumulative error which the subject exhibits in a visual tracking problem. In addition, this error can be sub-divided into contributions due to (1) prolongation of reaction time, (2) slowing of the maximum velocity of movement, and (3)

instability producing oscillations. Such an analysis is able to detect minor degrees of impairment of function. It is also capable of measuring this impairment in a quantitative and easily compared form. (see fig. 5 and 6).

DETAILED ANALYSIS OF THE SERVOCONTROL OF MOTION AT THE SPINAL CORD LEVEL AND PERIPHERALLY

Considerable work has been done in the experimental animal in studying the control of movement. Figure 4 illustrates the most reasonable scheme by which these elements are interconnected. There are two major ways in which muscle contraction can be produced: The first is via the pyramidal system whereby the upper motor neuron excites the alpha motor neuron. These impulses are carried directly to the extrafusal muscle fiber which shortens under this direct stimulus. The second mechanism is via the extrapyramidal route. Extrapyramidal impulses excite the gamma motor neuron which in turn produces shortening of the intrafusal muscle fibers. These fibers being weak do not produce shortening of the muscle itself but stretch the annulospiral endings. These spindle afferents in turn excite the alpha motor neuron producing a contraction of the extrafusal fiber which shortens the muscle as described obove. However, as the muscle shortens, the stretch on the annulospiral ending is reduced. This reduces the stimulus to the extrafusal fiber. Thus, equilibrium is once again achieved at a new shorter length.

It is presently believed that most of our voluntary motor activity is not transmitted via the pyramidal system directly. For example, skilled and highly organized movements such as those involved in driving an automobile are not conscious separate movements but involve a smooth flow of patterned effort which is only under the general direction of the consciousness. These differences are most clearly seen in comparing the driving performance of a beginner with those of an experienced driver. The beginner's performance is a series of voluntary willed acts which are performed in a jerky and isolated fashion when compared with the performance of the skilled driver.

The detailed functioning of this system was studied in man by recording two output parameters: (1) motor unit activity of the extrafusal muscle fiber as measured by intramuscular needle electrodes, (2) isometric muscle tension as measured by strain gauge. The experimental conditions may be varied in one of four ways: (1) the degree of base line voluntary activity which the subject exerts, (2) an abrupt lengthening of the muscle produced by a quick pull, (3) an abrupt shortening of the muscle produced by a quick release, (4) an abrupt synchronous firing of all muscle fibers produced by an electrical stimulus applied to the motor nerve (fig. 4, S).

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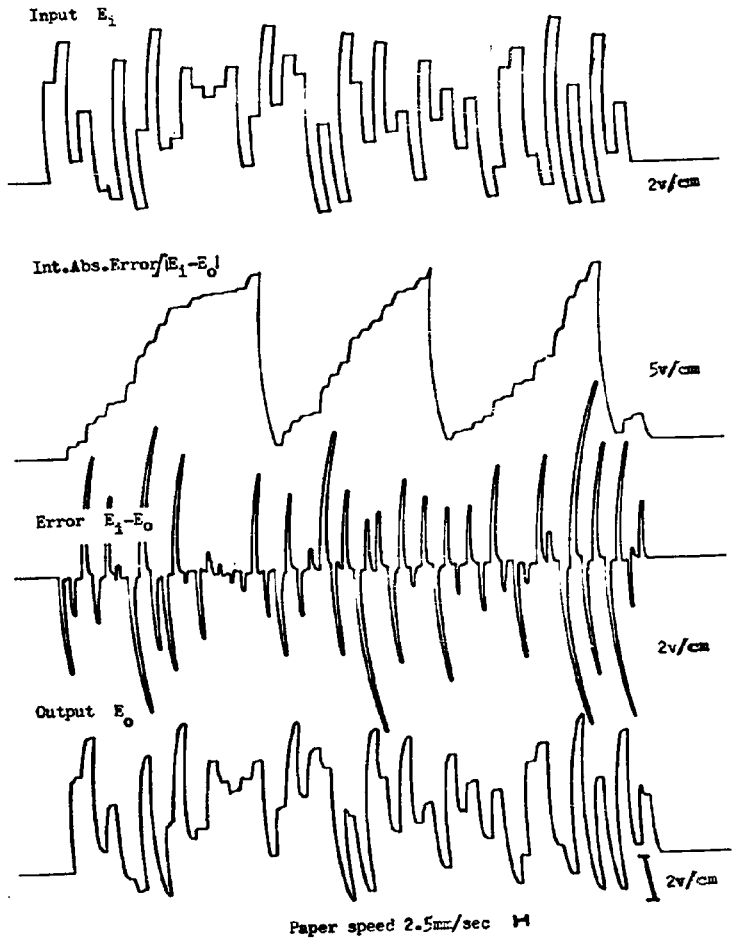

The top line is the position of the input signal light as a function of time (see fig. 2). The bottom line is the output signal, or pointer position, as a function of time. The third line is the difference between the input and the output, the error. On the second line is recorded the intergral of the absolute value of the error signal. This represents a summation of the total error which the subject produces.

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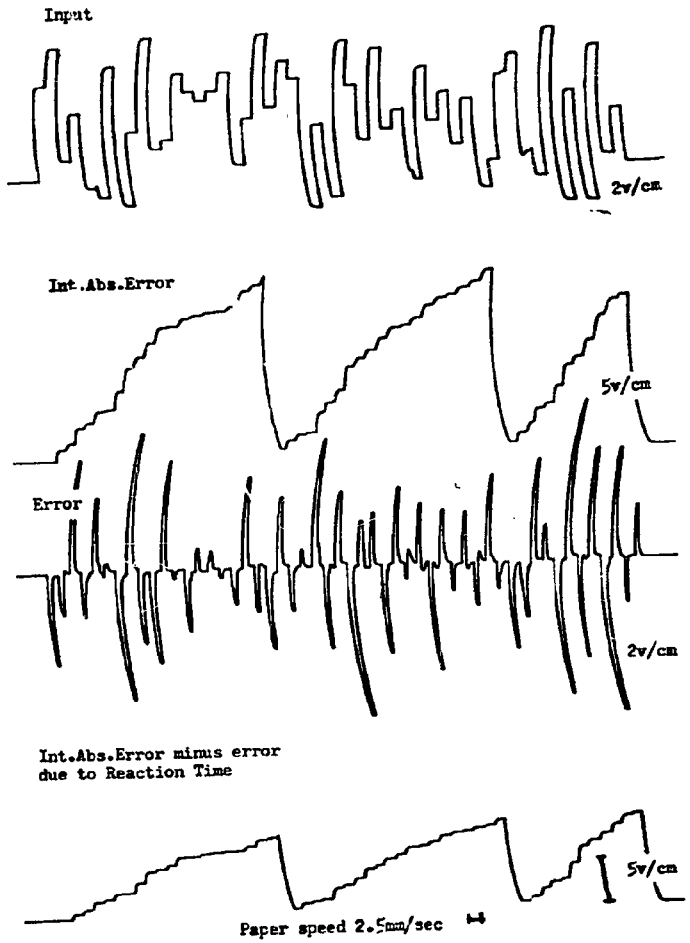


Here is shown a second run on the same normal subject. On the bottom line is recorded the total error less the error due to the reaction time of the subject.

A single example of the type of information obtained by this form of study relates to distinguishing between effects of stretching the annulospiral ending and the effects of stretching the Golgi tendon organ. Table 1 lists the effect on alpha motor neuron firing produced by various stimuli which would be expected on the basis of Figure 4. The results obtained in normal subjects are underlined. These findings indicate that the effect of quick pull is mediated by the Golgi tendon organ while that of quick release is via the annulospiral endings. The effect of nerve volley can be explained on either basis.

TABLE 1

	GOLGI TENDON ORGAN		ANNULOSPIRAL ENDINGS	
Stimulus	Effect	Alpha Firing	Effect	Alpha Firing
quick pull	stretch	<u>reduce</u>	stretch	increase
quick release	relax	increase (?)	relax	<u>reduce</u>
nerve volley	stretch	<u>reduce</u>	relax	<u>reduce</u>

Such detailed analysis of disordered movement is only warranted when preliminary studies have indicated that the defect lies in this area. Current investigations are limited to the disordered movement in parkinsonism. It is anticipated that studies will be extended to the drug induced dyskinesia, including the parkinson-like syndromes produced by phenothiazine derivative.

In 1956 several cases were reported in which there was a familial reduction in plasma cholinesterase (ChE) [1,2]. Attention was drawn to their defect only because of prolonged apnea which followed the administration of succinylcholine at surgery or electroshock therapy. Apart from this biochemical sensitivity to succinylcholine, these patients were healthy, free of liver disease, and had normal levels of red blood cell ChE.

The present case was found to have profound reduction in his red blood cell ChE activity, while his plasma ChE activity was normal. But for the unique circumstance of his occupation, the defect would not have been discovered, for he was otherwise in good health.

CASE REPORT

A.R. (JHH #1021997) was a 44 year old white male referred for investigation of low RBC ChE activity. Apart from usual childhood illnesses, tonsillectomy at age 5, a fracture of the right arm at age 12, and episodes of hay fever in the month of August, the patient had enjoyed excellent health.

The patient was employed in a supervisory capacity at a chemical plant engaged in the manufacture of organophosphorous anticholinesterase compounds. In the Spring of 1961, prior to any possible exposure to anticholinesterases, his RBC and plasma ChE levels were said to be normal (Table 1). In October, 1961 he was found to have low RBC ChE activity. Although the patient had no known exposure to anticholinesterase compounds and had no symptoms suggesting exposure, minimal inadvertant exposure was suspected and steps were taken to prevent any possible further exposure. During the following month considerable fluctuation in his RBC ChE level was reported. The patient stated that the laboratory was experiencing technical difficulties. Because his RBC ChE did not return toward normal, the patient was referred for evaluation in February, 1962.

On physical examination temperature, pulse, and respirations were all normal. Blood pressure was 115/70. The patient was stocky and slightly obese. No abnormalities were found on careful examination. In particular, there were no physical signs of anticholinesterase intoxication.

Except for a persistantly low RBC ChE activity of 0.29 - 0.32 ΔpH/hr (normal range 0.79 $\pm$ 0.10 ΔpH/hr, mean $\pm$ S.D.), the results of laboratory studies were well within normal limits, including plasma ChE activity of 0.82 - 0.87 ΔpH/hr, (normal range 0.88 $\pm$ 0.17 ΔpH/hr, mean $\pm$ S.D.), and a Ham test for acid hemolysis. The patient was discharged on no treatment.

He was admitted for further study in May, 1962. Although there had been no opportunity for exposure to anticholinesterase compounds, his RBC ChE activity remained low (0.24 - 0.32 ΔpH/hr). A family study was undertaken which indicated a familial basis for this defect.

FAMILY STUDIES

RBC and plasma ChE determinations were performed on the patient's parents and siblings (Table 2). One sister's RBC ChE was depressed to a similar degree, while his mother's level was less depressed. The RBC ChE was normal in the others and the plasma ChE activity was normal in all.

Detailed blood groupings were performed by Dr. Fred H. Allen, Jr. Assuming dominant inheritance there were linkage scores for ABO, Duffy, and haptoglobin with re-combinations in the ABO and haptoglobin systems, but not Duffy. An x-linked inheritance could not be excluded on present data.

The parents came from separate Lithuanian Jewish communities, and believed the possibility of cousin marriage was unlikely. None of the family had experienced serious illness, nor had any undergone surgery since the introduction of succinylcholine.

CHOLINESTERASE STUDIES

A number of studies were performed on the RBC ChE of the propositus, and in some instances of his family.

Methods and Materials. ChE activity was determined by the electrometric method of Michel[3]. Acetylcholine iodide was substituted for the chloride in equimolar concentrations, and no saponin was used. All determinations were performed in triplicate with appropriate concurrent controls for non-enzymatic acetylcholine hydrolysis and non-enzymatic acid formation.

ChE inhibition was accomplished by incubation of the hemolysed RBC suspension at 25°C. with inhibitor for 30 minutes in the final concentrations noted. The inhibitors used were isoflurophate (DFP), neostigmine methylsulfate, choline chloride, procaine hydrochloride, and dibucaine hydrochloride.

In the in vitro studies of oxime the hemolysed RBC suspension was incubated with the stated final concentrations of pyridine aldoxime methochloride (PAM·Cl) for 30 minutes prior to ChE determination. In the in vivo studies PAM·Cl was given in solution by mouth.

Effect on normal ChE. To test for the presence of an inhibitor of RBC ChE or a substance interfering with the determination, the patient's hemolysed RBC suspension was mixed with a normal hemolysate in known proportions (Table 3). The observed activities agreed closely with those calculated on the basis of simple admixture. No evidence of an inhibitor or interfering substance in the patient's RBC was found.

Effect of oxime. Certain oximes including PAM·Cl are capable of restoring ChE activity after inhibition, particularly when inhibited by organophosphorous inhibitors such as DFP. This restoration of ChE activity can be demonstrated in vitro [4,5] and in patients whose ChE has been inhibited[6,7,8].

PAM·Cl was added in vitro to the patient's RBC ChE and to normal ChE inhibited by DFP (Table 4). While the oxime produced significant increases in the DFP inhibited ChE, there was no increase in the patient's RBC ChE activity. The mild anticholinesterase effect of PAM·Cl itself was seen as a slight reduction in the normal control ChE activity.

The in vivo administration of PAM·Cl produced no increase in RBC ChE activity (Table 5). The administration of 3 grams daily for 3 days were similarly without effect. Thus, oxime in amounts sufficient to reverse organophosphorous inhibition was without effect in this patient.

Inhibitors. In order to detect qualitative differences between the patient's residual RBC ChE activity and normal RBC ChE activity both were subjected to inhibition by anticholinesterases. The inhibitors differed widely in potency, mode of action, and chemical structure. No significant abnormalities were found in the susceptibility of the patient's ChE to inhibition (Fig. 1). Dibucaine hydrochloride produced no RBC ChE inhibition at $5 \cdot 10^{-3}$M and was insoluble at pH 8 in higher concentrations.

DISCUSSION

Anticholinesterase intoxication seemed unlikely due to the absence of known exposure, absence of symptoms, and failure of his RBC ChE to rise after termination of all possible exposure. Since recovery of RBC ChE after irreversible inhibition depends upon the production of new red cells, the possibility of coincident toxic exposure and arrested erythropoiesis was considered. This was quickly excluded for no anemia had developed, yet the persistant RBC ChE depression would have demanded cessation of erythropoiesis for several months.

While many hematologic conditions are associated with modest reductions in RBC ChE activity[9], only in paroxysmal nocturnal hemoglobinuria is the level reduced to this degree[10]. When this disease was excluded by Ham test, the patient was considered to have an unexplained reduction in RBC ChE activity.

Family studies supported the contention that the reduction in activity was not acquired. Further inquiry concerning the normal values reported in February, May, and September, 1961 revealed that the technician had been discharged for falsification of other data. Presumably these results were also incorrect.

The family studies suggested dominant inheritance, possibly x-linked. Further studies are under way on the next generation. The inhibitor studies discussed below have provided no basis for distinguishing the abnormal from the normal enzyme. Such a difference, as found in the plasma cholinesterase deficiency, might provide a biochemical method for detecting the heterozygous state.

The cholinesterases are a heterogeneous group of esterases which preferentially hydrolyze choline esters. They have been divided into two types according to substrate specificity and kinetics. Acetylcholinesterases (true, specific, or type I cholinesterases) are capable of hydrolyzing acetyl-β-methylcholine[11] and exhibit inhibition by excess substrate[11,12]. Cholinesterases (pseudo, non-specific, or type II cholinesterases) do not hydrolyse acetyl-β-methylcholine and show no substrate inhibition[11,12,13].

In addition to the differences in substrate specificity, there are important differences in inhibitor sensitivity and differences in the biological distribution of the two types[14]. In man the RBC, muscle, and nervous system contain acetylcholinesterase while the plasma contains a non-specific cholinesterase synthesized in the liver. Thus, while the familial reduction in RBC ChE activity may have superficial resemblance to the plasma ChE deficiency previously reported[1,2,15,16,17], there is no reason to suppose biological, biochemical, or genetic similarities between these defects.

The human plasma ChE deficiency is manifest in its homozygous form by increased sensitivity to succinylcholine which depends upon ChE for its rapid destruction and short action[1,2]. This abnormal ChE had increased sensitivity to inhibition by dibucaine[15,16,17] and had recently been separated from the normal esterase chromatographically and electrophoretically[18]. These distinctive biochemical and physicochemical features permitted identification of asymptomatic heterozygotes having admixtures of normal and

abnormal esterase. Such distinction has not yet been possible with the RBC ChE defect.

The functional role of acetylcholinesterase in the red cell is not clear. Most[10,14,19] but not all[11,20] workers find virtually all RBC ChE activity in the stromal fraction of the hemolysate. While increased RBC permeability following anticholinesterase exposure has been found[21], it does not necessarily follow that the permeability changes were due to ChE inhibition, or that ChE maintains normal permeability. Similarly, the correlation of reduced RBC ChE activity and structural defects in the RBC stroma in paroxysmal nocturnal hemoglobinuria[10] may simply be associated phenomena. Indeed, the present observations, the absence of acetylcholinesterase in the RBC of some animals[14,22], and the normal survival of inhibited red cells[23] cast some doubt on a direct association between esterase activity and structural integrity of the RBC stroma.

SUMMARY

1. A patient was described whose red blood cell cholinesterase activity was reduced to one-third normal. There were no associated symptoms or signs, and the case was discovered because of routine cholinesterase determinations.

2. The patient's mother and sister were similarly affected.

3. No biochemical characteristics of this abnormal cholinesterase have been found which permit it to be distinguished from the normal enzyme.

TABLE 1

REPORTED CHOLINESTERASE LEVELS

Date	RBC ChE ΔpH/hr	Plasma ChE ΔpH/hr	Comment
8 Feb 61	0.72	0.98	
23 Feb 61	0.73	1.02	
18 May 61	0.75	0.98	
15 Sept 61	0.79	0.76	
10 Oct 61	0.40	0.90	exposure suspected
17 Oct 61	0.32	0.85	
18 Oct 61	0.25	0.50	control
	0.45	0.93	2 hrs after PAM 1 gm p.o.
	0.35	0.85	4 hrs after PAM 1 gm p.o.
19 Oct 61	0.39		control
	0.50		after PAM 1 gm p.o.
24 Oct 61	0.31	0.71	
25 Oct 61	0.31	0.71	
26 Oct 61	0.63	0.69	
30 Oct 61	0.80	0.77	
27 Nov 61	0.12		old reagents
	0.89		new reagents
26 Dec 61	0.40		
28 Dec 61	0.45		
29 Dec 61	0.39		
2 Jan 62	0.42		
4 Jan 62	0.37		
11 Jan 62	0.40		
18 Jan 62	0.39		
25 Jan 62	0.36		
1 Feb 62	0.38		
3 Feb 62	0.39		

TABLE 2

CHOLINESTERASE ACTIVITY IN KINDRED

Subject	Age	Relation	RBC ChE ΔpH/hr	Plasma ChE ΔpH/hr
L.R.	72	Father	0.58	0.70
P.R	71	Mother	0.45	0.64
M.B.	45	Sister	0.29	0.64
A.R.	44	Propositus	0.24	0.67
J.R.	39	Brother	0.60	0.88
R.P.	35	Sister	0.60	0.80

TABLE 3

ADMIXTURE OF PATIENT'S AND NORMAL RBC CHOLINESTERASES

Proportion		Observed	Calculated
Patient	Control	ΔpH/hr	ΔpH/hr
3/3	0	0.32	—
2/3	1/3	0.46	0.45
1/3	2/3	0.58	0.57
0	3/3	0.70	—

TABLE 4

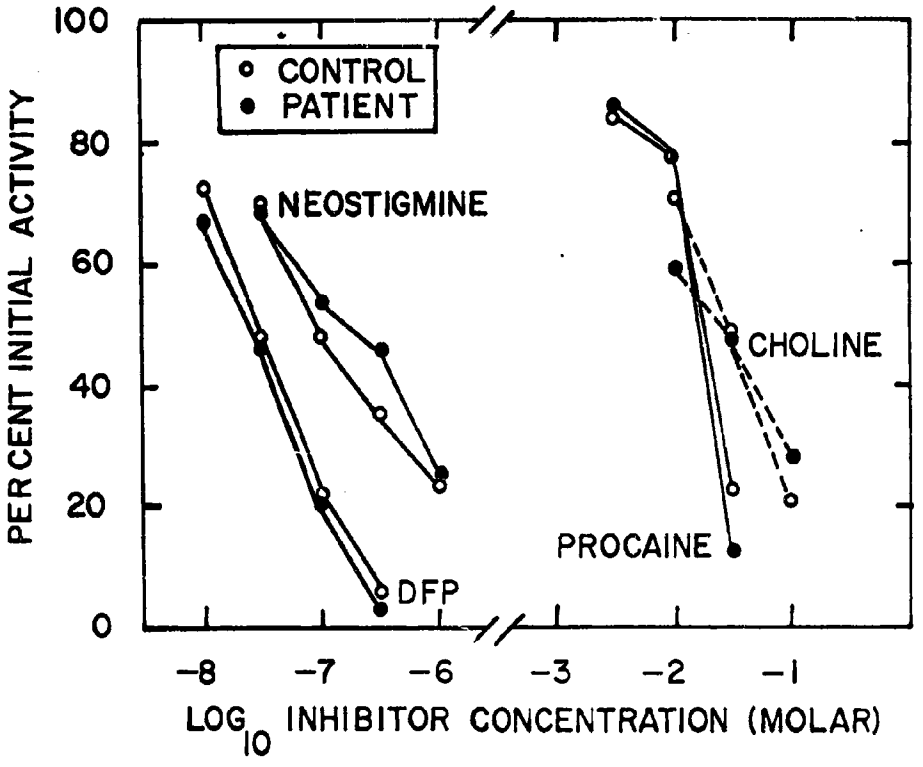
IN VITRO EFFECT OF OXIME ON RED BLOOD CELL CHOLINESTERASE ACTIVITY

PAM·Cl conc.	Patient		Control		Control DFP $5 \cdot 10^{-7}$M	
	ΔpH/hr	Per cent	ΔpH/hr	Per cent	ΔpH/hr	Per cent
0	0.28	100	0.60	100	0.12	100
10^{-5}M	0.20	71	0.52	87	0.14	117
10^{-4}M	0.28	100	0.53	88	0.18	150
10^{-3}M	0.23	82	0.52	87	0.42	350

TABLE 5

IN VIVO EFFECT OF OXIME ON RED BLOOD CELL CHOLINESTERASE ACTIVITY

Time	RBC ChE ΔpH/hr	PAM·Cl gm
9 a.m.	0.28	2
10 a.m.	0.30	
11 a.m.	0.30	
12 a.m.	0.29	



LEGENDS

Fig. 1 Comparison of the effect of various inhibitors on the patient's (closed circles) and normal (open circles) RBC ChE activity. Initial activities: patient, 0.28 to 0.30; normal control, 0.69 to 0.80 ΔpH/hr.

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Part III: Titrimetric Cholinesterase Method

The patient described in the preceding section stimulated interest in devising a simple method for studying the kinetics of cholinesterase. The Michel method is not well suited to this type of study, inasmuch as the time interval over which observations must be made (1 hour) is long with respect to the intervals of interest. Furthermore, it is not readily adaptable to the use of other substrates . The Warburg technique shared the disadvantage that observations of reaction velocity can be made only at ten minute intervals.

For these reasons a simple red cell cholinesterase method was devised using commercial equipment (Radiometer).

METHOD

Equipment Used:

Radiometer Titrator TTT1C
Radiometer Titrigraph SBR2C

Settings: (instructions followed for pH Stat)

Titrator: Selector - up scale
End Point - pH 8.00
Proportional band - 0.1 pH
Temperature - 20°C
Shut off delay - ∞

Titrigraph: Recording - pH Stat
Compensation - 0
Sample temperature - room temperature
Chart Calibration (x:1cm = 1 min; y:1div. = 1% of syringe volume)
A = 2.5, B = 4, C = 30, D = 1
Syringe burette - 0.5 ml

Reagents:

0.15 M NaCl
0.05 M NaOH (approx)
0.01 M NaOH (Fisher #So-S-284)
0.11 M ACh I (302 mg/10 ml)
RBC twice washed in saline and packed

Procedure:

1. Dilute washed packed RBC 1:100 in saline (add 1 drop of 1% saponin if not hemolysed)
2. Pipette 5 ml hemolysed RBC solution into pH cup
3. Adjust pH to about 7.8 or 7.9 with 0.05 M NaOH
4. Put sample on titrator and titrate pH to 8.00 exactly using 0.01M NaOH
5. Add 0.5 ml of 0.11 M ACh I
6. Titrate for 6-8 minutes
7. Run in duplicate
8. Run non-ACh acid formation control (no ACh)
9. Run non-enzymatic ACh hydrolysis control (no RBC)

Calculations:

Activity = μEq acid produced per minute per ml of packed RBC suspension

$$= \frac{\mu Eq\ NaOH\ used}{min \times ml.\ of\ packed\ RBC\ suspension}$$

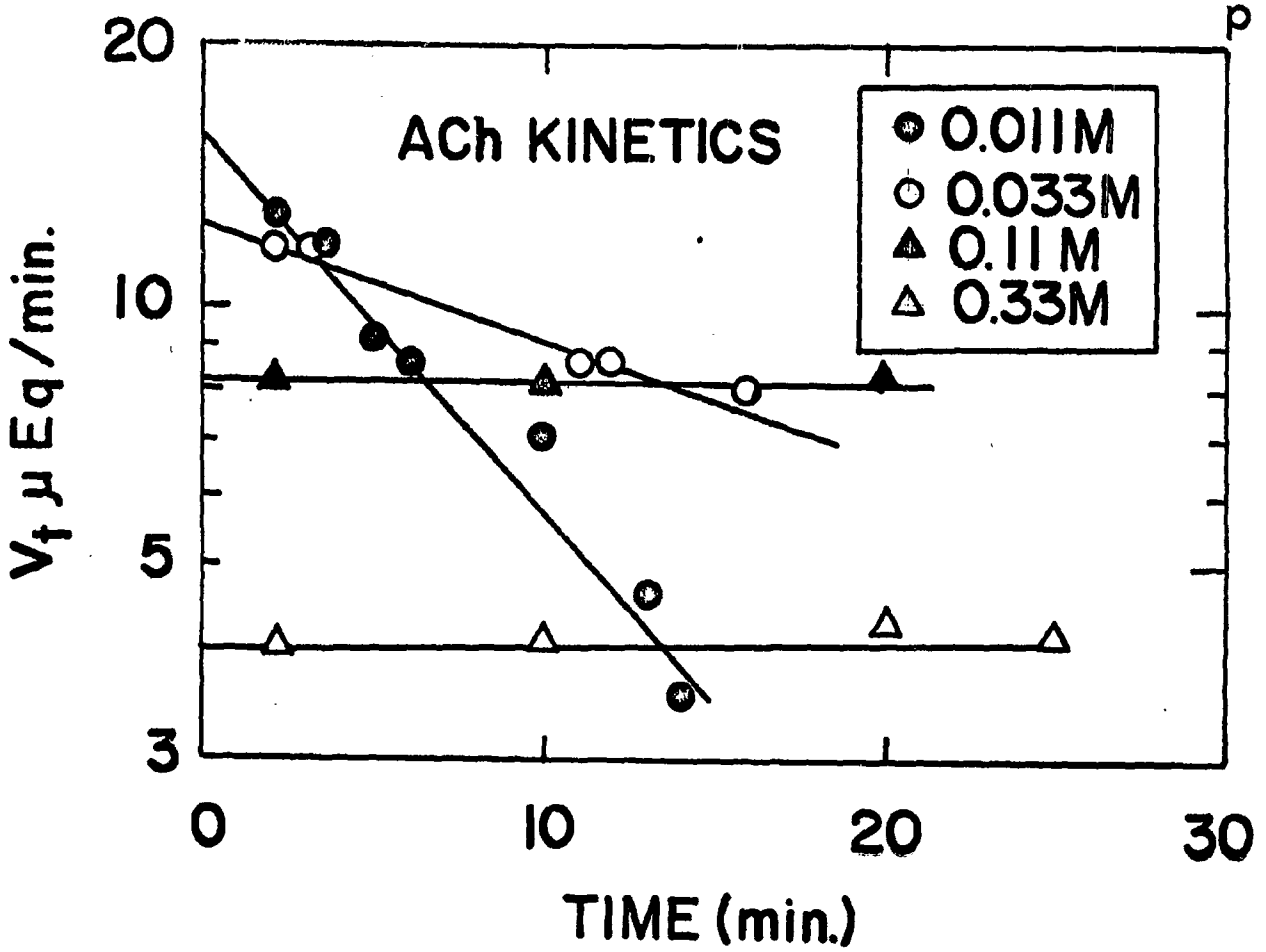
$$= \frac{y \times 0.01 \times 5}{min \times 0.05}$$, where y is the number of chart divisions, and the reaction mixture is as specified

= y/minute

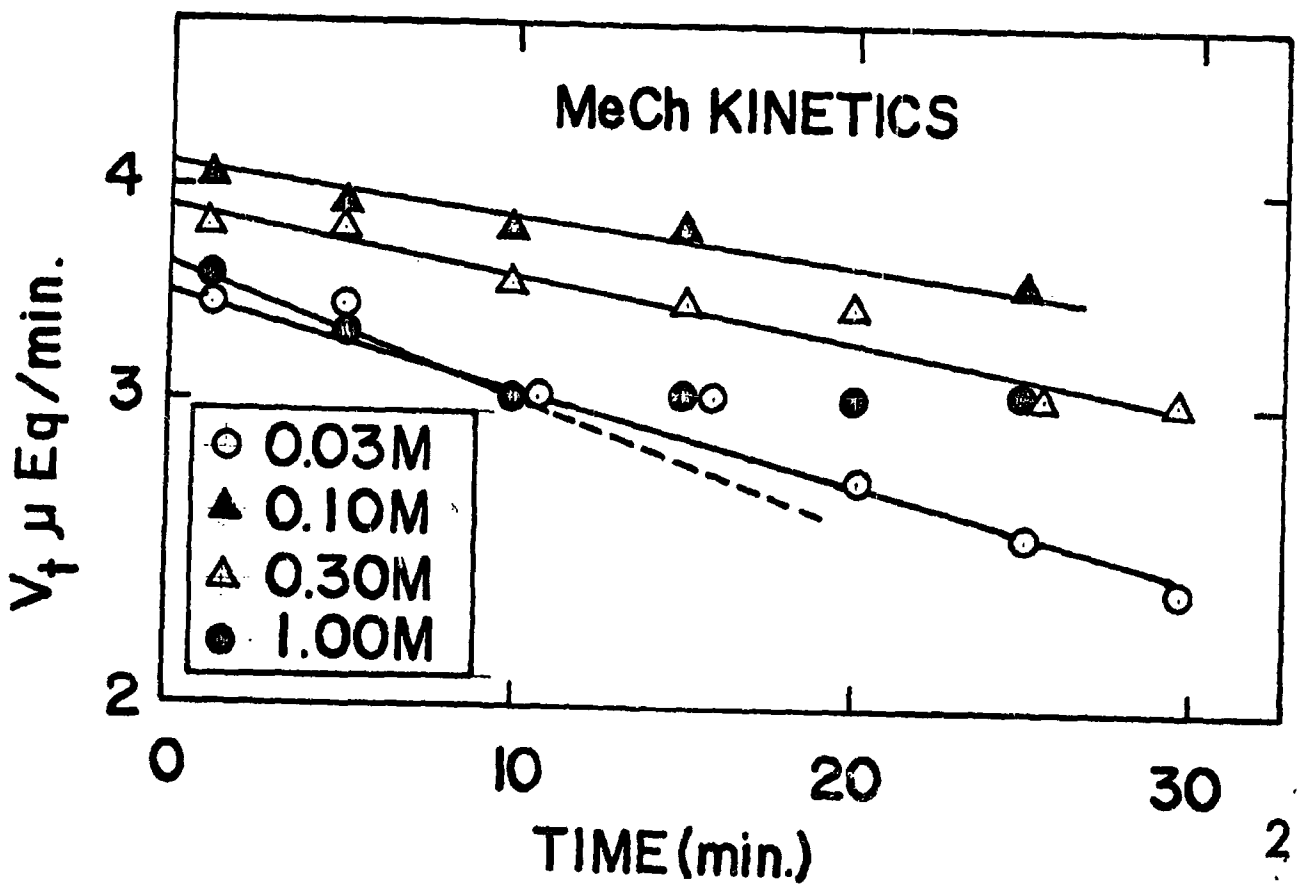
Results: Preliminary experiments were performed to determine the optimum concentration of acetylcholine. In figure 1 reaction velocity, V_t, is plotted as a function of time at several substrate concentrations. It can be seen that at concentrations of 0.033 M and below the reaction velocity decreases with the passage of time, although initial velocities are greater. The extrapolated values for the initial velocity, V, are shown in a Lineweaver and Burke plot in figure 3. Although higher initial velocities were obtained at the lower substrate concentrations, an initial concentration of 0.11 M was chosen, since no reduction in velocity was observed over a thirty minute period.

Similar data were obtained for mecholyl, and these are illustrated in figure 2. The extrapolated values for the initial velocity are plotted in figure 3. It can be seen that the maximum velocity is lower than that for acetylcholine, and at all concentrations reduction in reaction velocity with the passage of time is observed. The optimum concentrations of mecholyl was found to be 0.1 M.

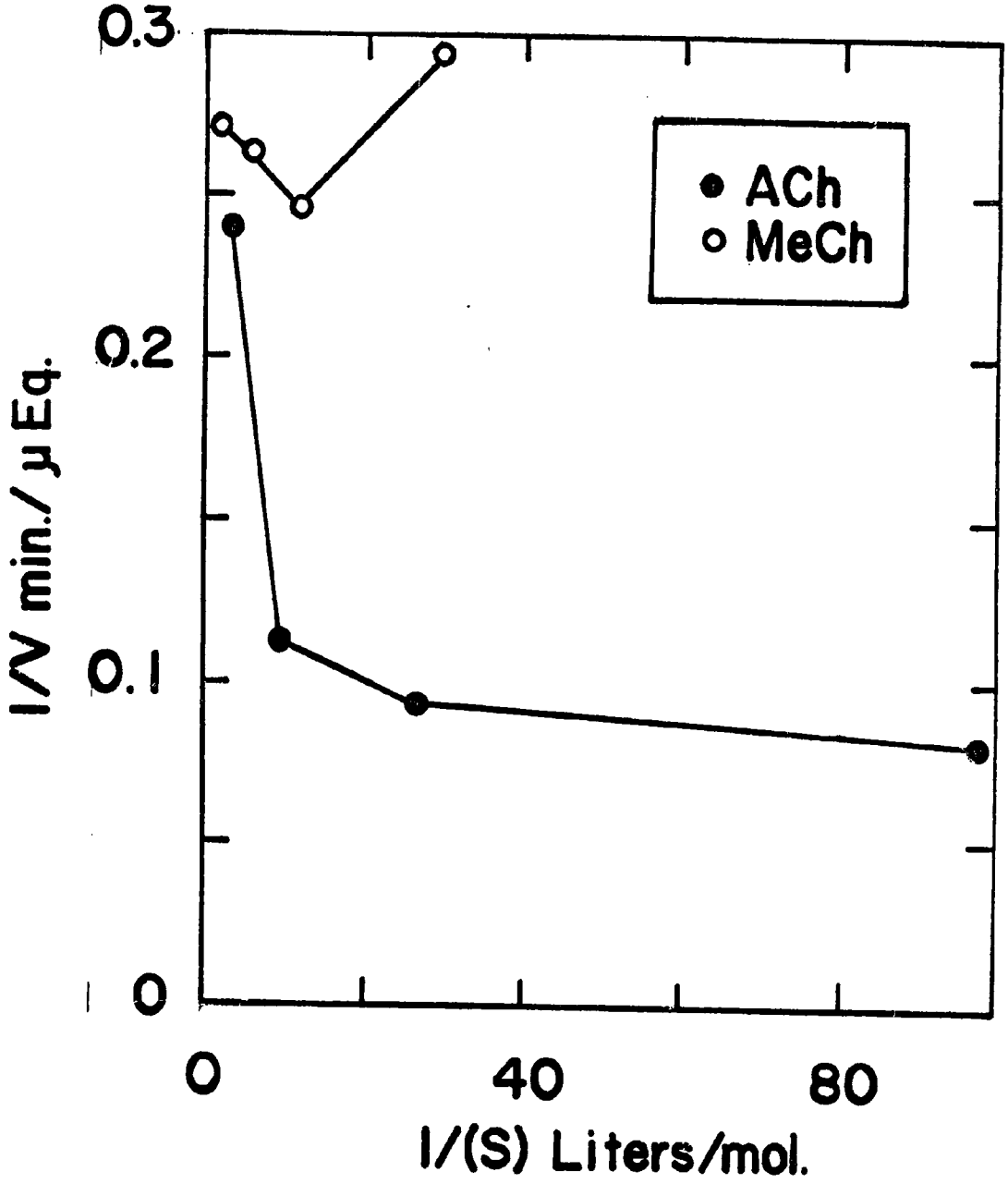
Comparisons were made between the present method and the Warburg method for determining cholinesterase activity using purified bovine RBC cholinesterase. With the present method one unit of cholinesterase produced 0.036 mM of acid per minute at 25° Centigrade, while using the Warburg method 0.045 mM of CO_2 were produced per minute at 37° Centigrade.


ACh KINETICS
0.011M
0.033M
0.11M
0.33M
V_t μ Eq/min.
20
10
5
3
0
10
20
30
TIME (min.)

1


MeCh KINETICS
0.03M
0.10M
0.30M
1.00M
V_t μ Eq/min.
4
3
2
0
10
20
30
TIME (min.)

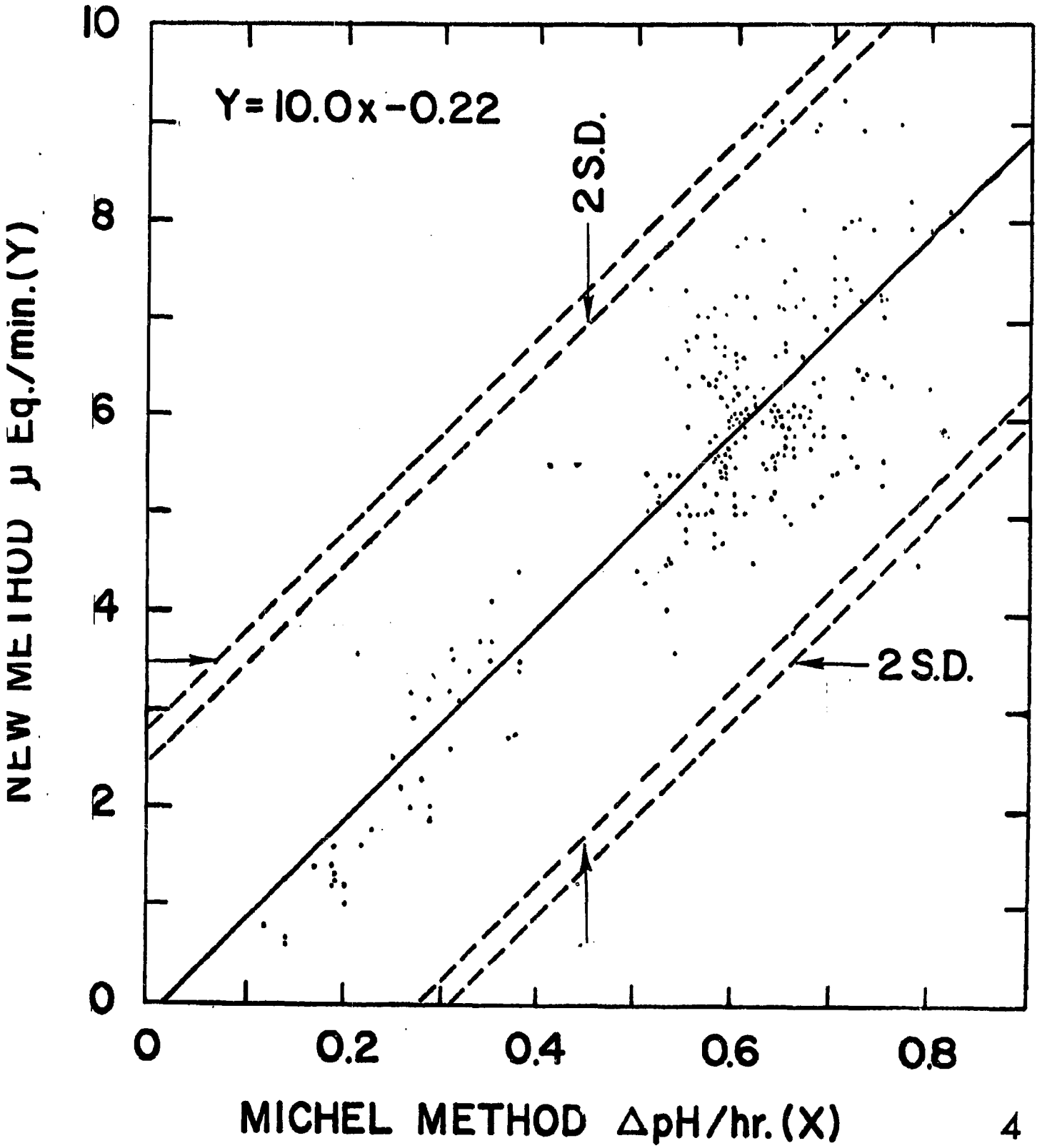
2


0.3
0.2
0.1
0
1/V min./μEq.
ACh
MeCh
0
40
80
1/(S) Liters/mol.

Comparisons were made between the present method and the Michel method on duplicate determinations of cholinesterase on red cells from 101 normal subjects and on blood of 20 normal subjects inhibited with DFP. The results of these studies are shown in Table 1 and figure 4. It can be seen that the Michel method has a lower coefficient of variance (.10) than does the present method (.20). Correlation between the two sets of data is reasonable (r = 0.71) .

TABLE I

	Present Method		Michel Method	
	μEq./min	%	ΔpH/hr.	%
number	202		202	
mean	6.1	100.	0.63	100.
standard deviation	1.2	20.	0.066	10.
standard error mean	0.088	1.4	0.0046	0.73
mean difference between pairs	0.14	2.3	0.03	4.8
standard deviation of difference between pairs	0.17	2.8	0.02	3.2


Y = 10.0x - 0.22
2 S.D.
2 S.D.
NEW METHOD μ Eq./min.(Y)
MICHEL METHOD ΔpH/hr.(X)
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8

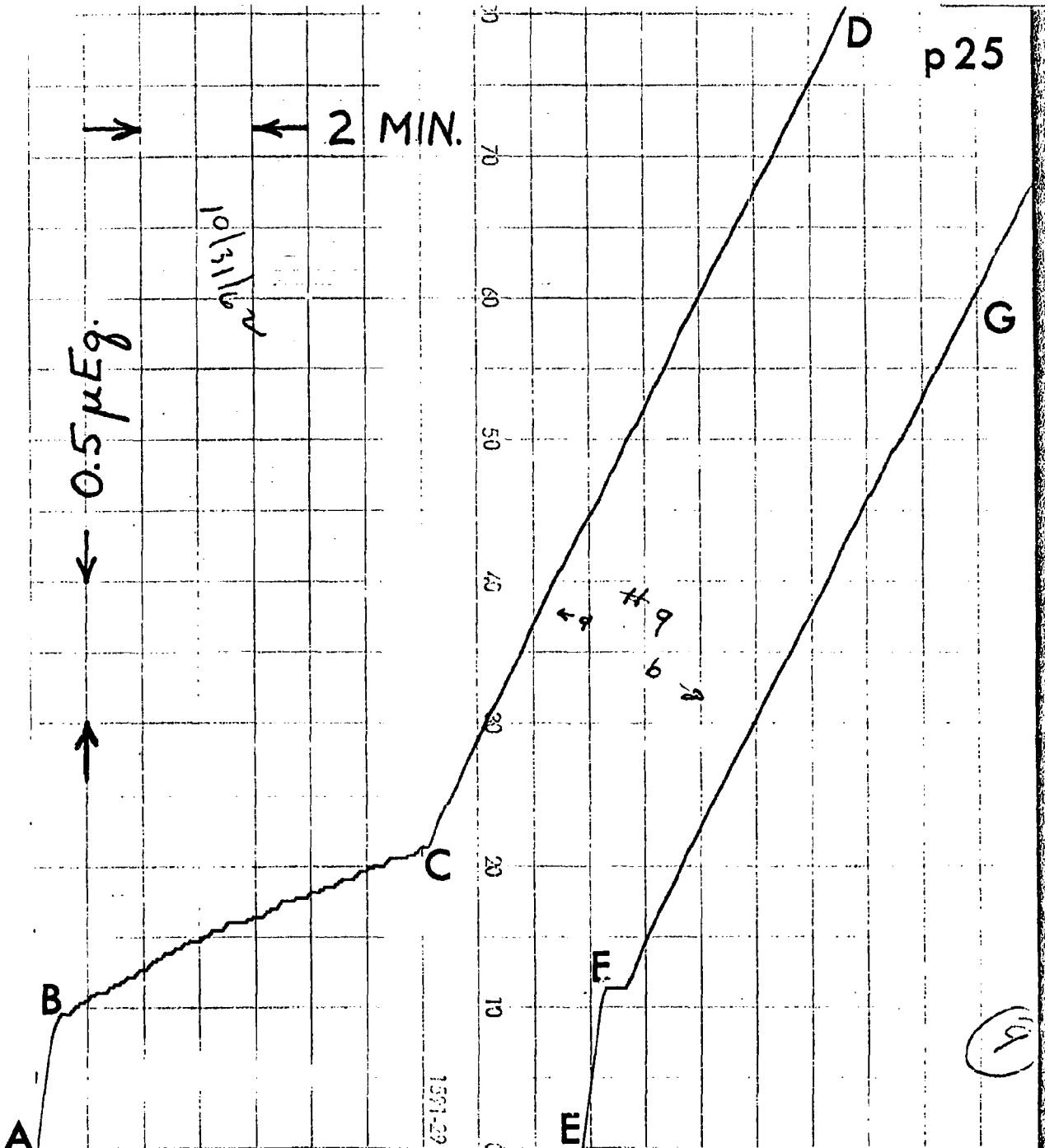


Figure 5. Ordinate: amount of NaOH added to maintain pH at 8.00. Abscissa: time. A-B is the titration of the reaction mixture to pH 8.00. The slope of B-C is the rate of non-ACh acid formation. At C ACh was added. The slope of C-D is the total rate of acid formation. E-F is the adjustment of the pH of a replicate to 8.00, while F-G is its reaction rate after the addition of ACh at F.

Part IV: Anticholinesterase Tolerance in Man

Since the brief report of this condition in the Annual Comprehensive Report covering 1 January 1961 - 31 December 1961 an additional patient exhibiting this syndrome has been discovered. She is in all essential features similar to the others previously described. Dose effect curves following pyridostigmine were obtained in which clinical strength, neuromuscular transmission, and RBC and serum cholinesterase were measured. Studies have been completed in the tolerant state. They will be repeated in a few months time when the tolerance has subsided.

These studies have been reported in detail in a paper submitted for clearance; this will be made a part of the next Annual Comprehensive Report. Results were summarized as follows:

1. A series of five asthenic women, without objective evidence of myasthenia gravis, tolerated massive amounts of anticholinesterase medications after a gradual increase in dosage, and yet were not dependent upon these drugs after withdrawal.

2. Certain features common to them were the presence of atypical weakness without fatigue on effort, bulbar symptoms yet no bulbar signs, various psychiatric problems, multiple system complaints, and definite improvement in strength after placebo.

3. Studies of neuromuscular transmission showed no block in transmission in the basal state, and no change after neostigmine.

4. Similar patients, described in the literature, were offered to support this condition as a recognizable and preventable syndrome.

5. While several mechanisms are possible, the precise nature of this tolerance remains unknown.

Part V: Anticholinesterase Tolerance in Rats

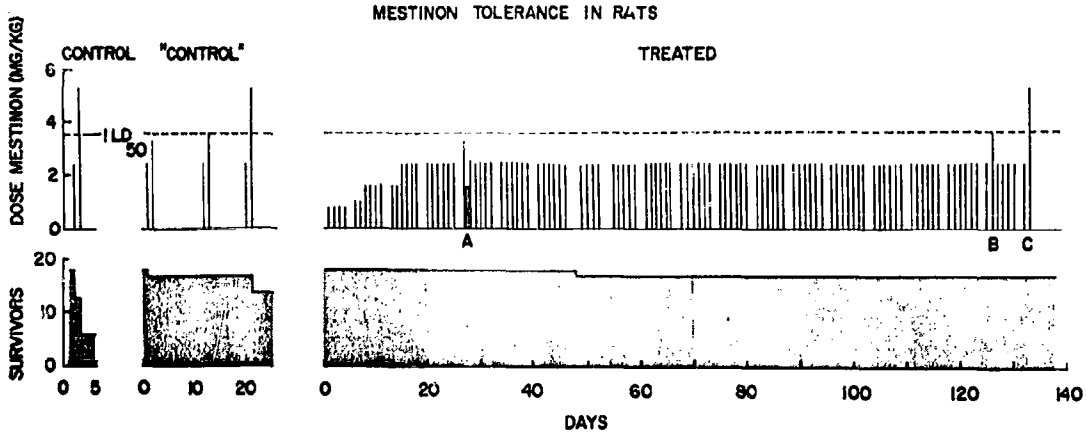
Introductory discussion and preliminary studies were reported in our recent Annual Comprehensive Report covering 1 January 1961 - 31 December 1961. In brief, we have shown that it is possible to produce a similar tolerance in animals to quarternary ammonium anticholinesterases (pyridostigmine) and to organophosphorous anticholinesterases (DFP, flurophate). The effect on RBC and plasma cholinesterase was compared with the effect in the normal control animal. Cholinesterase activity of the RBC and plasma of rats was determined by a modification of the method of Stubbs and Fales (Am. J. Med. Tech. 26, 25-32, 1960). The depth of the cholinesterase depression and its duration were measured by obtaining blood specimens just before, 1 hour after, and 5 hours after a 2.25 mg/kg injection of pyridostigmine. Results are summarized in Table 1.

Under control conditions there was no significant difference between the cholinesterase levels of the non-tolerant and tolerant animals. One hour following the administration of drug RBC cholinesterase was depressed to approximately 60% of normal. There was no significant difference between tolerant and non-tolerant animals. Similar findings were found with respect to plasma cholinesterase although the enzyme inhibition was greater. Five hours after drug administration the cholinesterase recovery of the tolerant animals was significantly ($P < 0.001$) slower than that of the non-tolerant rats.

If one is to assume that the observed tolerance is tolerance to anticholinesterase action, the present findings clearly indicate that this tolerance is not shared by the effect on RBC cholinesterase. This does not exclude, although it makes unlikely, the possibility that tolerant animals have less tissue cholinesterase depression than normals or that their tissue cholinesterase depression is of shorter duration than normal. This will be subjected to experimental tests in terminal experiments on these tolerant animals at which time tissue cholinesterase determinations will be performed.

These data make it more likely that the tolerance is to transmitter (ACh) or its action.

Figure 1 shows the protection against the lethal effect of pyridostigmine afforded by repeated administration of sub-lethal doses. In control experiments (left) it can be seen that 3/4 LD-50 produced 5 deaths and the administration of an additional 1 1/2 LD-50 on the following day resulted in a total of 12 deaths in a group of 18 animals. In contrast, a similar group of animals rendered tole-


MESTINON TOLERANCE IN RATS
CONTROL
"CONTROL"
TREATED
DOSE MESTINON (MG/KG)
6
4
2
0
1 LD50
A
B
C
SURVIVORS
20
10
0
0 5
0 10 20
0 20 40 60 80 100 120 140
DAYS

rant (right) by more than 120 days of pyridostigmine injection showed no deaths upon administration of 3/4 LD-50 (at C) or on the subsequent injection of 1 1/2 LD-50 on the following day. That this tolerance may develop quite rapidly is suggested by preliminary "control" studies (center). Here, as few as five sub-lethal injections afforded considerable protection to the administration of 1 1/2 LD-50 of pyridostigmine, the total deaths being only 3/18 compared to 12/18 in the control group.

Current studies include an identical design utilizing DFP treated rats. In addition, the development of tolerance to the effect of transmitter substance (ACh) itself are under way. Here, a depolarizing drug was chosen which is not influenced by the presence or absence of normal cholinesterase activity; decamethonium.

TABLE 1

ANTICHOLINESTERASE EFFECT OF MESTINON IN TOLERANT AND NON-TOLERANT RATS

	n	INITIAL ΔpH/hr mean	INITIAL per cent mean ±SEM	1 HOUR AFTER ΔpH/hr mean	1 HOUR AFTER per cent mean ±SEM	5 HOURS AFTER ΔpH/hr mean	5 HOURS AFTER per cent mean ±SEM
RBC ChE							
Non-tolerant	16	0.19	100±2.6	0.11	61±3.6	0.16	85±3.6
Tolerant	17	0.18	100±4.1	0.12	65±6.2	0.12	67±4.9
P_{diff}		>0.2			>0.2		<0.001
Plasma ChE							
Non-tolerant	17	0.40	100±2.9	0.17	41±2.2	0.34	83±3.0
Tolerant	17	0.40	100±3.8	0.15	38±2.5	0.23	58±4.5
P_{diff}		>0.2			>0.2		< 0.001

Considerable difficulty has been encountered in the use of decamethonium as a drug to assess sensitivity to depolarization. Thus far we have been unable to produce a constant degree of block. The various techniques which we have used unsucessfully are listed below:

1. Inject a single dose to obtain approximately 50% block.
2. Inject a single dose to obtain approximately 50% block. Follow this with constant injection to maintain approximately 50% block.
3. Inject at a constant rate until a 50% block is obtained. Adjust rate of injection to maintain this level.
4. Giving loading dose which produces slight block. Begin constant injection. At a block of approximately 50% adjust rate of injection to maintain constant block.
5. Give minimum effective dose, wait until effect begins to show, then start constant injection apparatus and approach 50% block.
6. Calculate rate of injection which should maintain a 50% block. Begin constant injection at this rate. Leave at this rate with no adjustments.

With all of these the degree of block of neuromuscular transmission as measured by the size of the action potential in response to a supermaximal nerve stimulus would either increase or decrease. At no time did it remain constant for a sufficient period of time to permit detailed studies. Recently it became apparent that this lack of stability has been the experience of others as well (Paton, W. D. M., and Waud, D. R. Drug-receptor interactions at the neuromuscular junction, p. 34-47, and Zaimis, E. Experimental hazards and artefacts in the study of neuromuscular blocking drugs. p. 75-82, both in Ciba Foundation Study Group # 12 " Curare and Curare - like agents, " A.P.S. De Reuck, ed., Little, Brown and Company, Boston, 1962). For this reason we are returning to the direct microelectrode approach to the determination of sensitivity of end plates. Because of large qualitative differences in the in vivo and in vitro studies we are adapting this technique for use in vivo using rat intercostal muscle.